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Photorhabdus Luminescens Phase II Cells Growth Kinetic Study Using A 5L A Plus Sartorius Stedim Biostat® Fermentation System

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Abstract

Photorhabdus luminescens lives symbiotically with the nematode species *Heterorhabditis bacteriophora*. This symbiotic couple may become a bio-control key to replacing chemical pesticides. The nematode is able to infect a wide variety of destructive insects without causing harm to beneficial insect species. There are numerous advantages of biocontrol methods including decreased maintenance and less repeated use than chemical pesticides. Nematodes are also resilient to the environment for reproduction. To better assess the growth characteristics of *Heterorhabditis bacteriophora*, the growth kinetics of the bacterial symbiont *Photorhabdus luminescens* must be understood. By varying the media composition, optimal conditions were found to present the highest specific growth rate and the shortest doubling time of *P. luminescens*. These conditions could be scaled into mass production with high yield.

Keywords: Photorhabdus luminescens, Heterorhabditis bacteriophora

Introduction

Photorhabdus luminescens is a gram negative, pigment producing, enteric bacterium that is capable of undergoing phase variation [1, 2]. *Photorhabdus luminescens* is bioluminescent and is pathogenic to insects (Figure 1) [1, 2]. This bacterium can switch between two epigenetic and metabolically different states; Phase I and Phase II. In Phase I, the metabolic state of *Photorhabdus luminescens* is bioluminescent and has red pigmentation. Phase I produces several "virulent factors" including: extracellular proteases, extracellular lipase, antibiotic substances, and intracellular protein crystals [3, 4]. Phase I metabolic state of this bacterium serves a key role in the symbiotic relationship with the nematode *Heterorhabditis bacteriophora*. Virulent factors which are produced during the nematodes' infective stage (IJ), kill the insect and create a nutrient hemolymph upon which the bacteria and nematodes feed [1,4]. After reproducing, the dead insect carcass bursts open and the infective nematodes emerge to seek out insect hosts. Phase II metabolic state is non-bioluminescent and does not produce the virulent factors (Figure 2) [1,4].

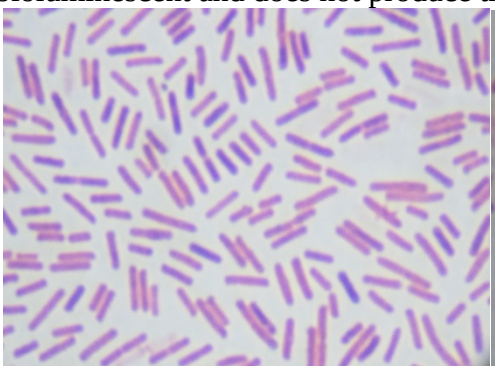

Figure 1: Gram-stain of *Photorhabdus luminescens* Phase I cells (1,000 x).

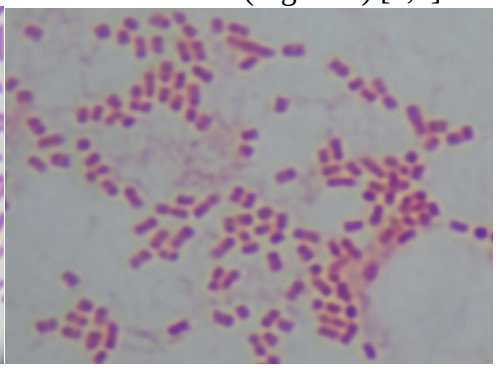

Figure 2: Gram-stain of *Photorhabdus luminescens* Phase II cells (1,000 x).

The microorganisms *P. luminescens* and *H. bacteriophora* have a complex symbiotic relationship. The nematode provides two main benefits: (1) protection from environmental conditions and (2) vector to insect host [2]. *Photorhabdus luminescens* grows in the gut of the nematode where it is protected. The nematode infects the target insects and releases the bacteria, which then reproduce [5].

This study shows media concentration effects on the growth kinetics of Phase II *Photorhabdus luminescens* using a 5 L Sartorius Stedim Biostat®

A plus Batch Fermentation System. By varying the composition of the media, optimal conditions for bacterial growth were found. Inoculating the bacteria into media and monitoring density over time, yields data sufficient for measuring bacterial growth. *P. luminescens* growth is displayed in four classical stages: lag phase, exponential phase, stationary phase, and finally, death phase [2]. Microbial growth kinetics measures the bacterial density during exponential phase to calculate the specific growth rate (SGR) and the doubling time (dt). Maximal specific growth rate indicates that conditions are optimal. [6,7]

Chemical pesticides can produce harmful effects on the environment [8]. Researchers are searching for replacements for chemical pesticides [9,10] which can affect all life forms. Research has found that beneficial nematodes target only insects that are parasitic to plants, and yet pose no threat to humans [11,12,13].

The purpose of this study is to understand the growth kinetics of Phase II *Photorhabdus luminescens* under different media concentrations using a Yoo media to determine the most optimal conditions for achieving the highest specific growth rate and the shortest doubling time. To the authors' knowledge, there has been scant research on Phase II cells of *Photorhabdus luminescens*. This paper pioneers Phase II behavior.

Materials and Methods

Bacterial Isolation and Scale-Up

To begin, the bacteria must be isolated and scaled-up for use [14]. The larva of wax moth *Galleria mellonella* were sanitized by submergence into 70% ethanol and dried on sterile filter paper [15]. Ten sanitized larva of *G. mellonella* were then introduced to infective juveniles of *H. bacteriophora* obtained from Arbico Organics® (Tucson, AZ USA) and incubated in the dark for 48 to 72 hours at room temperature [16]. After the incubation period, the *G. mellonella* were dead with red color indicating growth and reproduction of *H. bacteriophora* and *P. luminescens* [17,18]. The dead larvae were surface sanitized with alcohol and aseptically dissected using a sterilized wire loop to obtain hemolymph which was streaked onto nutrient agar (NA) plates. The plates were incubated at 28°C for 48 hours until large colonies appeared (Figure 3). To differentiate Phase I from Phase II cells, NBTA (nutrient agar bromothymol blue tetrazolium chloride agar) media plates were used. Red colonies indicate Phase II cells (Figure 4) [18]. NBTA contained per liter: 8.0 g nutrient agar; 25 mg bromothymol blue; 40 mg 2,3,5-triphenyltetrazolium chloride (TTC), nutrient agar contained per liter: 5 g peptone; 3 g beef extract; 15 g agar [16]. On a nutrient agar (NA) plate, red colonies and luminescence signals the presence of Phase I cells. A clear colony indicates Phase II cells. Isolated clear colonies of Phase II cells from

a NA plate were transferred into 50 ml 2x NB flasks and incubated at 28°C and 150 rpm for 24 hours. These 50 ml 2x NB flasks were the inoculum for the growth experiments [16].

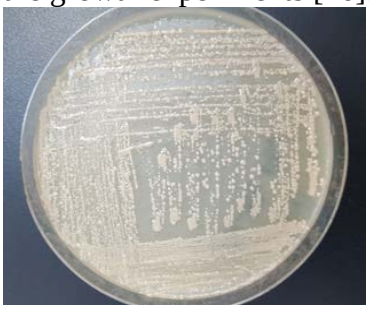

Figure 3: NA plate with isolated Phase II *Photorhabdus luminescens*

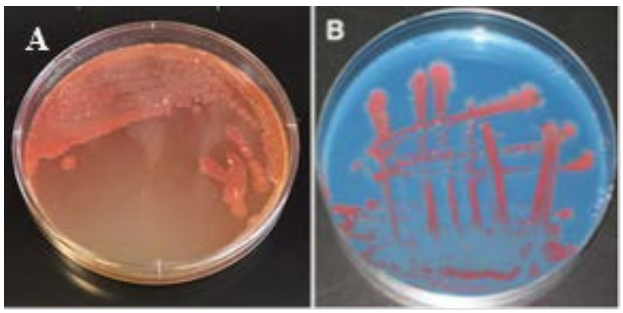

Figure 4: (A) *Photorhabdus luminescens* Phase I cells on NA plate (B) Red colonies of *Photorhabdus luminescens* Phase II cells on NBTA plate

Media Preparation

Stock Yoo media contains per liter: 25 g soytone; 5 g yeast extract; 10 g bacto-peptone; 200 mg cholesterol; 100 µl cod liver oil; 4 g NaCl; 5 g $MgSO_4$; 300 mg $CaCl_2$; 300 mg KCl; with pH adjusted to 7.5 [19].

Experimental Designs

This study was conducted in three designs with varying media composition (Table 1). The abiotic factors were set to a known standard optimal condition for every trial: temperature at 28°C, agitation rate at 300 rpm, air flow at 1 vvm, and pH 7.5 [20]. In Design 1, the concentration of soytone was varied: 0 %, 1.25 %, 2.5%, 3.75 % and 5 %. The yeast extract was set to 0.5 % and the peptone at 1 %. Other components of the media remained constant throughout every design. In Design 2, the concentration of yeast extract was varied: 0 %, 0.25 %, 0.5%, 0.75%, and 1 %. Soytone was kept at the optimal concentration determined in Design 1 and peptone was kept at its original concentration (1%). Lastly, in Design 3, the concentration of peptone was varied: 0 %, 0.5%, 1%, 1.5%, and 2 %. The soytone and yeast extract were set to optimal concentrations determined from Design 1 and Design 2.

Table 1: Experimental Designs

	Design 1	Design 2	Design 3
Soytone Variation Original 2.5%	0%, 1.25%, 2.5%, 3.75%, 5%	2.5% (Best Condition)	2.5%
Yeast Extract Variation Original 0.5%	0.5%	0%, 0.25%, 0.5%, 0.75%, 1%	0.5% (Best Condition)
Peptone Variation Original 1%	1%	1%	0%, 0.5%, 1%, 1.5%, 2% (1% Best Condition)

Measurement of Specific Growth Rates (SGR) and Doubling Times (DT)

Specific growth rate (SGR) is defined as the increase in bacterial cell mass per unit of time [21]. Doubling time measures the time needed for the bacteria to double in a culture. The Optek® Fermentor Probe in the reactor measures cell density in concentration units (CU). To determine the specific growth rate (SGR) for each experiment, the natural log (ln) of the concentration units (CU) is taken as a function of time (t) [2]. The Optek® probe records cell density at 10 min interval for 24 hours. The data was transformed to a scatter-plot graph and the specific growth rate and doubling time were determined (Figure 5). The slope of green markers on the ln(CU) line represents the determined SGR value, 1.36 h^{-1}.

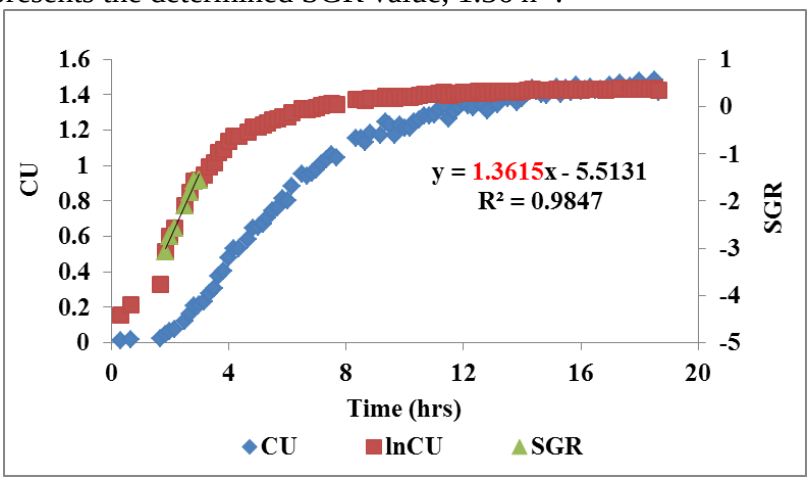

Figure 5: Determination of Specific Growth Rates (SGR)

Measurement of Bioluminescence

Photorhabdus luminescens Phase I is a bioluminescent bacterium, while Phase II shows no bioluminescence. A Turner Biosystems Modulus® was used to measure luminescence of a 1 ml sample. Lack of bioluminescence indicates the culture is in Phase II [22].

Result and Discussion

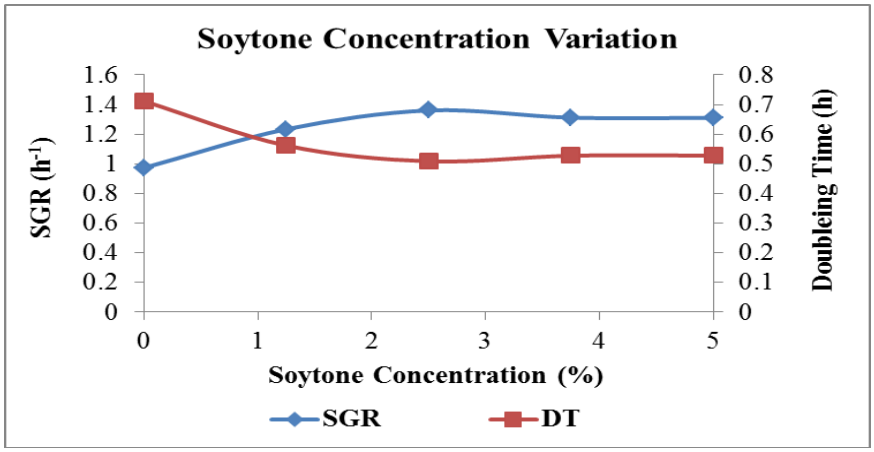

Figure 6: Effect of soytone concentration on SGR of *Photorhabdus luminescens* Phase II cells

This experiment was conducted in three series of experiments, changing the concentration of components in a Yoo media. These components were; 1) soytone, 2) yeast extract and 3) peptone. In the first design, the concentration of soytone was varied at: 0 %, 1.25 %, 2.5 %, 3.75 %, and 5 %. The original concentration of soytone (2.5%) was found to be the highest specific growth rate 1.4 (h^{-1}) and the lowest doubling time 0.51 (h). The soytone variation graph shows that the specific growth rate increases as it approaches the original media concentration and then decreases. SGRs slightly decreased as the concentration of soytone was increased from 2.5% to 5%. Islam et al. also reported a reduction in bacterial cell densities of *Bacillus subtilis* as the soytone concentration was increased from 1% to 2% during antibiotic production [23].

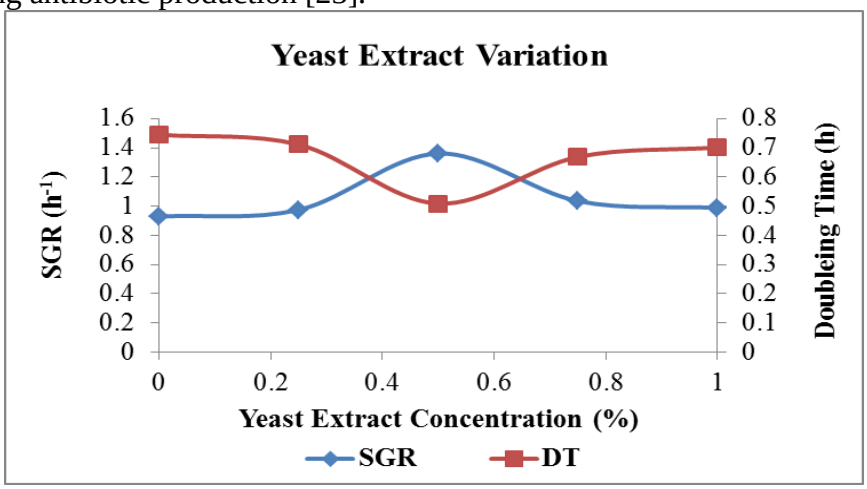

Figure 7: Effect of yeast extract concentration on SGR of *Photorhabdus luminescens* Phase II cells

Yeast extract is a mixture of amino acids, peptides, vitamins and carbohydrates and can be used as additive to culture media. In Design 2, the concentration of yeast extract was varied at: 0 %, 0.25 %, 0.5%, 0.75 %, and 1 %. The yeast extract variation data further supports the claim that the original Yoo media provides the most optimal concentration. The graph shows the same characteristic results at 0.5 % yeast extract, yielding a specific growth rate of 1.4 (h^{-1}) and a doubling time of 0.51 (h). The data indicates that the specific growth rate increases as it approaches the original media concentration and decreases at high concentration. High concentrations of substrate inhibit the growth and metabolism of microorganisms [24]. Amrane and Prigent reported SGR of *Lactobacillus sp.* was decreased from the 1.03 h^{-1} to 0.85 h^{-1} as they increased yeast extract concentration from 20 g/L to 30 g/L [25].

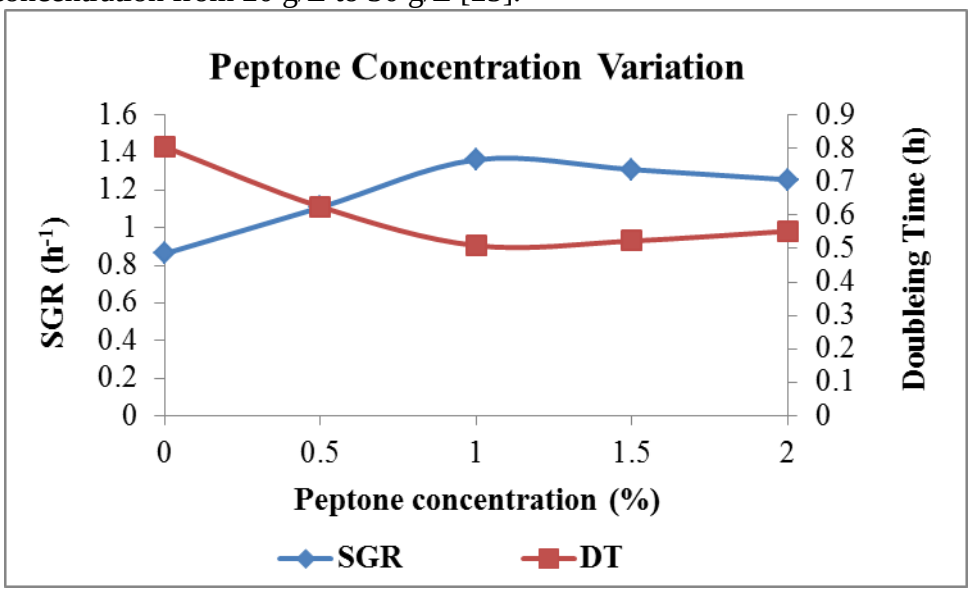

Figure 8: Effect of peptone concentration on SGR of *Photorhabdus luminescens* Phase II cells

Peptone is the principal source of organic nitrogen for the growing bacteria. The final series of experiments was variations in concentration of peptone in the Yoo media at: 0 %, 0.5 %, 1%, 1.5 %, and 2 %. Looking at the specific growth line for peptone variation in Figure 8, it can be seen that the original Yoo media with 1% peptone concentration provided the most optimal condition with specific growth rate of 1.4 (h^{-1}) and a doubling time of 0.51 (h). SGR declines as the peptone concentration was increased; due to substrate inhibition [Gray et al., 2008]. Amezaga and Booth reported that peptone component became limiting for the growth of *E. coli* in high osmolarity medium when cultures reached high cell densities [26]. The

peptone concentration variation graph shows the same characteristics as the soytone and yeast extract variations.

Han and Ehlers distinguished phase variants based on dye absorption, pigmentation, production of antibiotic substances, occurrence of crystalline inclusion proteins and bioluminescence. They also concluded that negative impact of Phase II symbiont cells on the development and reproduction of *H. bacteriophora* was not due to lack of essential nutrients or the production of toxins [3]. Ehlers et al. reported that substances responsible for the development of nematodes are produced only by Phase I *P. luminescens* [27]. Phase variations in *P. luminescens* are induced for adaptation to different environments such as high and low temperature, oxidative agents (H_2O_2), osmolarity, alkaline and acid conditions, and low oxygen supply [28]. Our investigations show that it is important to understand Phase II cells physiology under different media composition. Inman et al. conducted antibacterial screening of secreted compounds produced by the Phase I cells *P. luminescens* [22]. For future study, antimicrobial activity of this *P. luminescens* Phase II can be investigated.

Bacterial and nematode yield is determined by the concentration and composition of media components [29]. A media solution containing high sources of mono-unsaturated fatty acids and few saturated fatty acids is a requirement for optimal growth and development of *H. bacteriophora* and *P. luminescens*. Yoo et al. identified a suitable blend of olive and canola oil [19]. Nematodes use high lipid concentration for long term energy and food; the bacteria however have limited ability to convert mono-unsaturated fatty acids into usable energy. By increasing total bacterial cell mass within 24 hours, the time required for nematodes to become an infective juvenile decreases [19]. A shorter nematode incubation time will result in a more efficient, cost effective, and a more consistent quality. Optimal media conditions in the Yoo media for *Photorhabdus luminescens* will allow higher yield of *Heterorhabditis bacteriophora*.

Conclusion

This study was completed to understand *P. luminescens* Phase II cells and related microbial growth kinetics. This study investigated how *P. luminescens* Phase II cells respond to different concentrations of soytone, peptone and yeast extract of Yoo media in a bioreactor. Rapid cell growth is necessary for better yield during mass production in bioreactors [30]. Referring to the final results, the original Yoo media concentrations provided optimal specific growth rate yielding at 1.4 h^{-1} and the lowest respective doubling time of 0.51 h. The original Yoo media concentration provided the most suitable conditions for growing the largest bacterial cell mass of Phase II *Photorhabdus luminescens* in the shortest time. To carry out nematode

mass production successfully, an unintended phase shift to secondary phase should be avoided [31].

Acknowledgments

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